ACTIVATION OF RESPIRATION IN SEA URCHIN SPERMATOZOA BY EGG WATER[1]

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The fertilization of sea urchin eggs in sea water is preceded by interactions between the free-swimming spermatozoa and material that diffuses from the egg. The analysis of these interactions is of interest because of the likelihood that at least some of them are helpful or essential to fertilization. Attention has focused both on the soluble material of the egg, and on its various effects on spermatozoa (Metz, 1957; Wiese, 1961, for reviews); and in the present paper further observations of sea urchin spermatozoa in the presence of egg material are reported. Sea water containing material that has diffused from eggs is called "egg water," and in sea urchins, the best known component of egg water derives from the jelly that normally surrounds the egg. This "jelly-coat" is composed of a glycoprotein called fertilizin, which in solution causes the agglutination of sea urchin spermatozoa (Lillie, 1913; Tyler, 1956). Agglutination is thought to result from the formation of bonds between antifertilizin on the surface of spermatozoa, and fertilizin, each molecule of which is presumed to combine with two spermatozoa. The agglutination, however, is temporary or reversible, probably because of the swift degradation of fertilizin into a non-agglutinating or univalent form (Tyler, 1948; Hathaway and Metz, 1961).

Other reactions of sperm to egg water sometimes include (1) an enhancement of motility (Lillie, 1913, 1919), (2) changes in the respiratory rate (*e.g.*, Gray 1928b), (3) an acrosome reaction (Dan, 1952, 1956), and (4) a displacement of the mid-piece (Popa, 1927; Dan, 1954). The question arises whether these various reactions of spermatozoa can be attributed to fertilizin, or whether egg water contains other substances that affect the behavior and structure of spermatozoa. In some instances it is fertilizin, or a closely associated substance, that causes an increase in sperm motility. Fertilizin of *Strongylocentrotus purpuratus*, which had been precipitated and dialyzed, retained the property of activating motility (Tyler, 1955). In *Arbacia punctulata*, however, the activator of sperm motility differed from fertilizin by its volatility (Clowes and Bachman, 1921), and its diffusibility upon dialysis (Cornman, 1941). In *Echinocardium cordatum* the activator was also diffusible (Vasseur and Hagström, 1946). The activator in eggs of *Arbacia pustulosa* was reported to be the pigment echinochrome (Hartmann *et al.*, 1940), although this was questioned by Bielig and Dohrn (1950).

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Purified echinochrome did not activate sperm motility in *S. purpuratus* (Tyler, 1939), or in *A. punctulata* (Cornman, 1941). An extract of egg water from *Psammechinus miliaris* caused an increase in motility and respiration of spermatozoa, and the substance affecting motility was tentatively identified as vanillin (Lybing and Hagström, 1957).

On the whole, little is known of agents in egg water that affect the respiration of spermatozoa. Oxygen uptake of spermatozoa increases after the addition of egg water in *Echinus esculentus* (Gray, 1928b; Carter, 1931; Vasseur, 1949), and *Strongylocentrotus droebachiensis* (Vasseur, 1949). An increase in respiratory rate has also been reported in *Psammechinus miliaris* (Gray, 1928b; Carter, 1931; Vasseur, 1949). In the same species, egg water was also reported to cause a decrease in sperm respiration (Rothschild, 1952). A decline in respiratory rate was described in *Arbacia punctulata* (Hayashi, 1946), and in *Lytechinus pictus* (Spikes, 1949). In contrast, egg water had no effect on sperm respiration in *S. purpuratus* (Tyler, 1948). It is possible that these varied results were due to differences in the preparation and handling of egg water and spermatozoa before and during the experiments. Rothschild (1956a, 1956b) showed that the rate of sperm respiration was closely dependent on pH, and Mohri and Horiuchi (1961) found that the presence of respiratory CO_2 inhibits the utilization of endogenous phospholipid by spermatozoa, even in a buffered medium.

Experiments on sperm respiration are complicated by the phenomenon known as "Dilution Effect." Gray (1928a) observed that respiration was greater in dilute, than in dense, sperm suspensions, and that dilution of spermatozoa with sea water caused an increase in respiration. It has been suggested that Dilution Effect is due to copper in sea water (Rothschild and Tuft, 1950). This was supported by the demonstration that Dilution Effect is absent when a chelating agent is added to the diluent (Rothschild and Tyler, 1954).

The experiments to be described were initiated after the observation that *Arbacia* egg water caused an increase in sperm respiration, and that the respiratory activator was a substance that could be distinguished from fertilizin. Features of the interaction between the activator and spermatozoa were examined in three species of echinoderms. An electron microscope was used to examine possible changes in the structure of spermatozoa following activation. Some of the results have been described in a preliminary communication (Hathaway, 1960).

Materials and Methods

Arbacia punctulata was collected in the vicinity of the Florida State University Marine Laboratory, Alligator Harbor, Florida, or in some cases, near the Marine Biological Laboratory, Woods Hole, Massachusetts. Other species utilized in Florida were *Lytechinus variegatus* and *Mellita quinquiesperforata*. Eggs and semen were obtained from the gonopores after stimulation by electrical current or 0.55 *M* KCl solution. In both cases the gonads were stimulated directly through an incision in the peroral region. Semen was diluted in five volumes of filtered sea water buffered with glycylglycine, and centrifuged for five minutes at about 5000 *g*. The sedimented spermatozoa were resuspended in buffered sea water to ten times the volume of the semen. Aliquots of this suspension were introduced into the respirometer. Oxygen saturation of this suspension was evidently main-

tained in the respirometer flasks, since there was no marked increase in O_2 uptake after the additional agitation caused by a simulated tipping. The problem of O_2 saturation in dense suspensions of spermatozoa has been discussed by Rothschild (1956a). Dense suspensions were used in the present experiment to avoid Dilution Effect as far as possible.

Eggs were washed in several times their volume of sea water. Materials were obtained from washed eggs as follows: (1) Washed eggs were allowed to remain three hours in two volumes of sea water. The supernatant is referred to as "egg water." (2) The jelly-coats of the eggs were dissolved with sea water acidified to pH 4.0. The jellyless eggs were carefully washed in several changes of sea water, and then left for three to six hours in five volumes of sea water. The supernatant is referred to as "jellyless egg water." Temperatures were 20–22° C. during the procedures described above. (3) Five ml. of settled eggs were mixed with 25 ml. acidified alcohol (0.004 N HCl in ethanol). The deep red supernatant was dried *in vacuo* at 40° C., mixed with 15 ml. of sea water, and centrifuged. The supernatant was neutralized with NaOH, and the pH was adjusted to 7.99 with buffered sea water.

Glycylglycine at a final concentration of 0.02 *M* was used as a buffer (Tyler and Horowitz, 1937). The effects of chelation by glycylglycine have not been evaluated in this study; however, it would be expected to modify the influence of metals present in sea water. The pH values of solutions were measured with a Beckman Zeromatic meter.

Oxygen consumption was measured in a Warburg apparatus, with air as the gas phase in the flasks. The single-arm vessels of 15 to 20 ml. capacity contained one ml. of sperm suspension in the main compartment, 0.8 ml. of the experimental solution in the side arm, and 0.2 ml. of 10% KOH solution in the center well, which also contained a filter paper wick. Controls were run to obtain values for auto-oxidation, by substituting buffered sea water for the sperm suspension. The flasks were shaken at 120 cycles per minute, with an amplitude of 3.5 cm. Temperature was 20° C. in the Florida experiments, and 25° C. at Woods Hole. The sperm suspensions and the contents of the side arms were adjusted to equal pH values and mixed with buffer before they were placed in the flasks.

Results

Respiratory activation of spermatozoa by egg materials

Results in Table I show that *Arbacia* sperm respiration was initially increased three- to six-fold over controls by *Arbacia* egg water, jellyless egg water, acidic-alcohol extract, and the diffusate from a dialyzed homogenate of eggs. The initially high rates of respiration decreased by the end of the experiment ($2\frac{1}{2}$ or 5 hours), but were still higher than control values. Versene had the effect of maintaining a low but constant rate of sperm respiration. There was no auto-oxidation in any of the solutions. Almost constant levels of pH were maintained in the flasks. The slight fall in pH in some flasks was probably caused by the accumulation of CO_2. The results indicate that the increase in sperm respiration is due to agents that diffuse freely from eggs.

TABLE I

*Oxygen uptake of Arbacia spermatozoa**

Final concentration of glycylglycine = 0.02 *M*.
Sperm concentration = 1/10 × semen.
Initial pH of sperm suspension = 7.99.
Tipped at t = 40 minutes.

Solution tipped in and its pH	Duration of experiment, minutes	Rate of respiration 10 minutes after tipping: μl. O_2/hr.	Rate of respiration at end of experiment, μl.O_2 /hr.	Final pH of sperm suspension
Diffusate (dialysate) of homogenized *Arbacia* eggs 7.92	150	565	258	7.83
Acidic-alcohol extract 7.95	150	457	225	7.86
Sea water 7.98	150	92	45	7.98
Versene 2.5 × 10^{-4} *M* 7.99	150	59	60	8.03
Egg water 7.98	300	286	65	7.90
Jellyless egg water 7.96	300	302	102	7.85
Sea water 8.00	300	100	46	8.00

* Each entry in Tables I–V represents a single determination.

Some properties of activating solutions

The respiratory activator in egg water was lost during dialysis. Whereas undialyzed *Arbacia* egg water caused an immediate increase of 487% in *Arbacia* sperm respiration, the same egg water that had been dialyzed for 24 hours against running sea water had no effect. Although these results may indicate that the active agent is unstable in sea water, the loss of activity could have resulted from diffusion of the activator through the dialyzing membrane. As an additional test a dialysis bag containing 10 ml. of sea water was placed in a beaker containing a suspension of washed *Arbacia* eggs. After six hours at 20°, the contents of the bag and the supernatant sea water (egg water) in the beaker were tested separately for their effects on *Arbacia* spermatozoa. The respiratory rates increased by 254% and 337%, respectively. The diffusion of the activator through dialysis membrane indicates that it is not identical with fertilizin. The activator could be a low molecular weight derivative of fertilizin. If this were the case, the source of activator, like that of fertilizin, would be the jelly-coat of the egg. However, jellyless egg water, which does not agglutinate spermatozoa, is rich in activator, indicating that the source is probably the egg itself, rather than the jelly-coat.

It is known that the agglutination titer of fertilizin is reduced by reaction with spermatozoa. In order to test for a similar reduction in the strength of an activating solution, jellyless egg water was mixed with spermatozoa and left to interact for 90 minutes. The suspension was then centrifuged, and the supernatant solution was tested for activating property. Results in Table II show that the activating property was almost completely lost during the first exposure to sperm, suggesting a rapid absorption of the activator by the cells.

The motility of *Arbacia* spermatozoa was stimulated by a distillable product of *Arbacia* eggs (Clowes and Bachman, 1921). To determine if the respiratory activator has this property, an activating solution that caused an increase in sperm respiration of 987% was boiled under reflux at a pressure of one atmosphere for 20 minutes. The boiled solution stimulated sperm respiration by 950%. A distillate obtained from the activating solution was without stimulating effect on spermatozoa.

TABLE II

Decrease in the activating property of jellyless egg water during exposure to Arbacia spermatozoa

Solution tipped in	Per cent increase in the rate of O_2 uptake in the sperm suspension, measured 15 minutes after tipping
Jellyless egg water	646
Jellyless egg water exposed to *Arbacia* spermatozoa* for 90 minutes	55
Sea water	10
Sea water exposed to *Arbacia* spermatozoa* for 90 minutes	27

* Spermatozoa washed and diluted in same way as those used in the respirometer.

Jellyless egg water that caused a 917% increase in the rate of O_2 uptake of spermatozoa was extracted with an equal volume of n-butanol, and, after evaporation of the solvent, the residue was taken up in sea water. This solution activated sperm respiration by 750% whereas a butanol extract of sea water had no effect on spermatozoa.

Effects of amino acids upon the rate of sperm respiration

Amino acids and other chelating agents prolong the motility of sea urchin spermatozoa (Tyler, 1953), and stimulate the motility and respiration of starfish spermatozoa (Metz and Birky, 1955). Cysteine can stimulate (10^{-2} *M*), or depress (10^{-3} and 10^{-4} *M*) respiration in sea urchin spermatozoa (Mohri, 1956b). It seemed of interest to examine the effects of several amino acids on *Arbacia* spermatozoa. Only cysteine had a marked effect on sperm respiration (Table III), without, however, increasing sperm motility. The magnitude of the response of spermatozoa to cysteine suggested a comparison of some of the properties of the activator and cysteine. Solutions of cysteine at concentrations capable of stimulating sperm respiration yielded colored spots when tested on paper with ninhydrin, but jellyless egg water showed no trace of color when treated in the

TABLE III

Effect of amino acids on Arbacia sperm respiration*

Solution tipped in	Per cent increase in the rate of O_2 uptake by the sperm suspension measured 15 minutes after tipping
Histidine	120
Cysteine	1270
Glycine	53
Glutamic acid	10
Tryptophan	54
Arginine	10
Lysine	43
Sea water	56

* 2×10^{-3} *M* amino acid in buffered sea water.

same manner. Next, jellyless egg water, and a solution of cysteine (4.4×10^{-3} *M*) were each mixed with an equal volume of 4.4×10^{-3} *M* of the sulfhydryl reagent, iodoacetamide. At this concentration, iodoacetamide alone reduced the respiration of spermatozoa to almost nil within 45 minutes. In the mixture with jellyless egg water, iodoacetamide still reduced sperm respiration in 45 minutes, the egg water failing to abolish the inhibitory effect of iodoacetamide. In the mixture with cysteine, however, the inhibitory effect of iodoacetamide was abolished (Table IV). This suggests that the activator in egg water is not cysteine.

In this experiment, the stimulatory effect of cysteine was not diminished by iodoacetamide, and it may be that the sulfhydryl group is not essential for the stimulation of sperm by cysteine. This question was not pursued.

The activator also differs from cysteine in its effect on unbuffered suspensions of spermatozoa of the starfish *Asterias forbesi*. Jellyless egg water from *Arbacia* caused no increase in the respiration of *Asterias* spermatozoa, while 2.2×10^{-2} *M* cysteine caused an increase of 358%. In the same experiment, 2.2×10^{-4} *M* Versene caused an increase of 425% in sperm respiration. The activating action of cysteine in this case was probably a result of its chelating property (Metz and Birky, 1955).

TABLE IV

Effects of Arbacia jellyless egg water, cysteine, and iodoacetamide on the respiration of Arbacia spermatozoa

Final concentration of cysteine and iodoacetamide = 2.2×10^{-3} *M*

Solution tipped in	Respiratory rate before tipping: µl. O_2/hr.	Respiratory rate one hour after tipping; µl. O_2/hr.
Sea water	79	60
Sea water plus iodoacetamide	75	22
Jellyless egg water plus sea water	78	325
Jellyless egg water plus iodoacetamide	78	10
Cysteine plus sea water	82	225
Cysteine plus iodoacetamide	75	250

Species specificity of respiratory activators

Although the agglutination of spermatozoa by egg water is highly specific (Lillie, 1919; Metz, 1945; Tyler, 1948), there has been no clear demonstration of specificity among activators of sperm motility and respiration. A specific activator was thought to occur in starfish egg water (Loeb, 1915), but it was later demonstrated that starfish spermatozoa are virtually immotile in both sea water and egg water (Metz, 1945). In the same paper, Loeb also reported a strong cross-reaction in the activation of sea urchin spermatozoa by egg water. In view of these reports, gametes of several species were examined for respiratory activators.

The non-agglutinating, jellyless egg water from *Lytechinus variegatus* activated the respiration of *Lytechinus* spermatozoa. In a specificity test involving simultaneous observations on *Lytechinus* and *Arbacia* spermatozoa, the activators

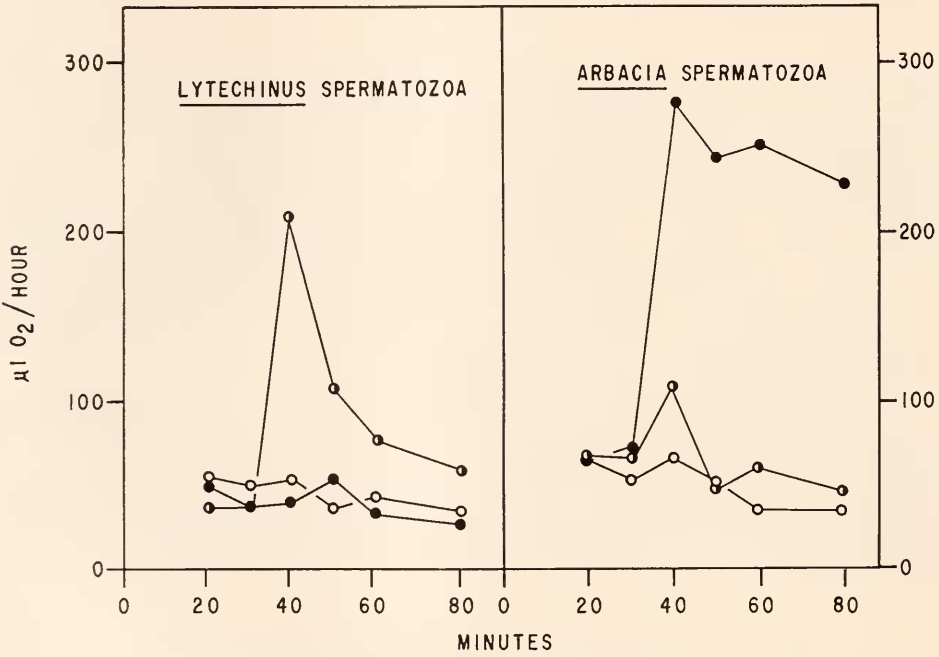

FIGURE 1. Respiratory rates of *Lytechinus* and *Arbacia* spermatozoa in a specificity test of activating solutions. Activating solutions tipped in at t = 32 min.: *Arbacia* jellyless egg water ●, *Lytechinus* jellyless egg water ◑, Sea water ○.

proved to be species-specific (Fig. 1). This experiment was performed on April 24, 1960. On two other occasions earlier in April, virtually the same results were obtained in similar experiments. A month later, on May 29, 1960, when *Lytechinus* was approaching the height of its reproductive activity, its spermatozoa displayed a strong respiratory response to *Arbacia* activator. However, a complete test of specificity could not be performed at that date.

The jelly-coats of *Mellita quinquiesperforata* contain numerous red granules which can be liberated with acidified sea water. These granules, when washed

and suspended in sea water, stimulated respiration of the spermatozoa of *Mellita* and *Arbacia*. In a simultaneous test, *Arbacia* activator had no effect on *Mellita* spermatozoa (Table V).

Sperm motility

Arbacia spermatozoa, which were moderately active in sea water, became intensely motile when mixed with egg water, jellyless egg water, and acidic-alcohol extract of *Arbacia* eggs, and with suspensions of the jelly-coat granules of *Mellita*. The jelly-coat granules also stimulated the motility of *Mellita* spermatozoa. Concentrations of cysteine that stimulated respiration had no effect on the motility of *Arbacia* sperm. *Lytechinus* spermatozoa were relatively unaffected by solutions prepared from specific eggs or from *Arbacia* eggs.

Other effects of jellyless egg water on spermatozoa

Numerous investigations have demonstrated that sea urchin spermatozoa undergo visible structural changes in the presence of material from eggs. Popa (1927) described the appearance of "lateral bodies" after exposure of *Arbacia*

TABLE V

Specificity of activators from Arbacia and Mellita

Solution tipped in	Sperm	Per cent increase in O_2 uptake by the sperm suspension measured 15 minutes after tipping
Arbacia jellyless egg water	*Arbacia*	1660
	Mellita	75
Suspension of jelly-coat granules from *Mellita*	*Arbacia*	1640
	Mellita	285
Sea water	*Arbacia*	10
	Mellita	78

spermatozoa to egg water and vital stains. More recent authors have interpreted the "lateral body" as a displaced middle piece, which appears when spermatozoa are treated with egg water, sea water deficient in calcium, or homogenates of eggs (Tyler, 1952; Dan, 1954; Bernstein, 1959). In addition, echinoderm spermatozoa frequently undergo an acrosome reaction in the presence of egg water (Dan, 1952, 1956; Metz and Morrill, 1955), and *Arbacia* spermatozoa have reacted acrosomes following treatment with purified fertilizin (Piatigorsky and Austin, 1962).

Arbacia spermatozoa were mixed with jellyless egg water, or with sea water, and fixed 20 minutes later in 1% formalin in sea water. Whole-mounts of these cells were examined in an electron microscope at a magnification of 7300. The proportion of displaced middle pieces was small and almost the same in the suspensions treated with activator (12.2% of 465 cells), and in suspensions mixed with sea water (11.4% of 484 cells). Reacted acrosomes were not seen, however; it is common with *Arbacia* spermatozoa to fail in the stimulation of the acrosome

reaction by methods (high pH, calcium-free medium, treatment with purified fertilizin) that are usually effective in other species.

Arbacia spermatozoa mixed with egg water release complex materials containing sialic acid (Warren, Hathaway and Flaks, 1960). This phenomenon appears to result from the interaction between spermatozoa and fertilizin, and the evidence suggests that sialic acid is a component of sperm antifertilizin (Hathaway, 1961). It was of some interest, therefore, to observe that *Arbacia* spermatozoa failed to release materials containing sialic acid during the interaction with the respiratory activator in jellyless egg water.

Discussion

The responses of sea urchin spermatozoa to products of eggs are similar in many respects to effects achieved with a variety of other agents of known or unknown composition. The foremost of these effects is an immediate change in oxygen uptake by dense suspensions of spermatozoa, caused by additives ranging from sea water (as in Dilution Effect), and hydrogen ions (pH effect), to uncoupling agents (Rothschild, 1956a), and sulfhydryl reagents (Barron and Goldinger, 1941). In a study of the effects of sodium azide, Mohri (1956a, 1956b) implicated the cytochrome-cytochrome oxidase system in the increased respiration that follows dilution of sperm with sea water, and he showed that the respiratory rate in dilute suspension is equalled in dense suspensions that are supplied with substrates (e.g., dimethyl-p-phenylenediamine) for the cytochrome system. More recently, dithiocarbamates have been found to cause an augmentation of sperm respiration, an effect that is cancelled by the presence of sodium azide (Muramatsu, 1963a, 1963b). Dermal secretions from *Arbacia* stimulate sperm respiration, as well as inhibit fertilization and sperm agglutination (Metz, 1959, 1960, 1961). A similar increase in respiration is found in sperm treated with another inhibitor of fertilization extracted from the alga *Fucus* (Branham and Metz, 1962).

The effect of slight changes in pH on respiration of spermatozoa has been studied by Rothschild (1956a, 1956b). In experiments with *Paracentrotus lividus* and *Echinus esculentus* this author observed a 400% increase in O_2 uptake when the pH value increased from 7.84 to 8.00. Since it is almost impossible to avoid slight changes in pH with dilution, Rothschild warns of the hazard of interpreting respiratory increases as Dilution Effect. This applies equally to interpretations of the effects of various substances on sperm respiration. The situation is different in *Pseudocentrotus depressus*, where spermatozoa undergo only small changes in respiration over a broad range of pH (Mohri and Horiuchi, 1961). These experiments duplicated the conditions used by Rothschild, and it is reasonable to conclude that wide variations exist in the responses of sea urchin spermatozoa to changes in pH.

Several things indicate that the respiratory responses of spermatozoa reported in this paper are not due merely to changes in pH. The pH values of suspensions were not elevated when they were checked at a stage when respiratory rates were still high. In fact, the pH was slightly lower in these suspensions because of the high rate of CO_2 production. Moreover, there was no marked increase

in respiration when sea water, rather than egg water, was added, in spite of the fact that it would have been just as difficult to avoid slight changes in pH when adding sea water. The consistent response of spermatozoa to egg materials, and the lack of response to sea water, argue against the interpretation that slight, uncontrollable variations in pH account for the results.

An activator of sperm motility was described in *Arbacia* eggs some time ago (Clowes and Bachman, 1921), but the enhancement of respiration by egg materials has apparently not been observed before in this genus. On the contrary, egg water has been reported to cause a decrease in O_2 uptakes by *Arbacia* spermatozoa (Hayashi, 1946). Hayashi observed a decline in the respiratory rate after addition of egg water to a dilute suspension of spermatozoa. This suspension had already experienced a burst of respiratory activity after dilution with sea water. Under such conditions, O_2 uptake was apparently curtailed by the agglutination of the spermatozoa.

The identity of the respiratory activator, and the mechanism of its action upon spermatozoa, remain unknown. The activator is not identical with fertilizin, since it is diffusible during dialysis, and soluble in acidified ethanol and butanol. Also, it fails to agglutinate spermatozoa, and does not induce the release of sialic acid from these cells. The possibility has not been excluded that the activator is a low molecular weight derivative of fertilizin (*cf.* Tyler, 1955). The fact that this agent is readily obtained from jellyless eggs suggests that it is not simply a breakdown product of fertilizin. This is consistent with the observation that sperm motility is increased by an agent that diffuses from jellyless eggs (Loeb, 1915).

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Summary

1. Sea urchin gametes were used in measurements of the effect of egg materials upon sperm respiration.

2. Dense suspension of spermatozoa of *Arbacia punctulata* had a relatively low rate of oxygen uptake in buffered sea water. After the addition of egg water or materials diffusing from jellyless eggs (jellyless egg water), there was a marked increase in respiratory rates. A similar effect was seen after the addition of cysteine, but not after the addition of several other amino acids, Versene, or buffered sea water. It was concluded that *Arbacia* eggs contain an activator of sperm respiration.

3. Solutions lost their activating properties during interaction with spermatozoa. The respiratory activator diffused through dialyzing membrane, and was alcohol-soluble, heat-stable, and non-volatile. It differed from cysteine in its re-

action with ninhydrin and iodoacetamide. It failed to activate respiration in starfish spermatozoa, unlike cysteine and Versene, which stimulated respiration in these cells. The activating agent in question remains unidentified, but it is evidently neither fertilizin nor cysteine.

4. Respiration of spermatozoa of *Lytechinus variegatus* was increased by diffusates from *Lytechinus* eggs. A species specificity exists in the respiratory activators of *Lytechinus* and *Arbacia*.

5. Suspensions of the red granules from the jelly-coat of *Mellita quinquiesperforata* activated the respiration of spermatozoa of both *Mellita* and *Arbacia*. Diffusates from *Arbacia* eggs failed to affect *Mellita* sperm.

6. No structural changes were observed in *Arbacia* spermatozoa incubated with activating solutions. Unlike fertilizin, the activator in jellyless egg water failed to cause the release of sialic acid from sperm.

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